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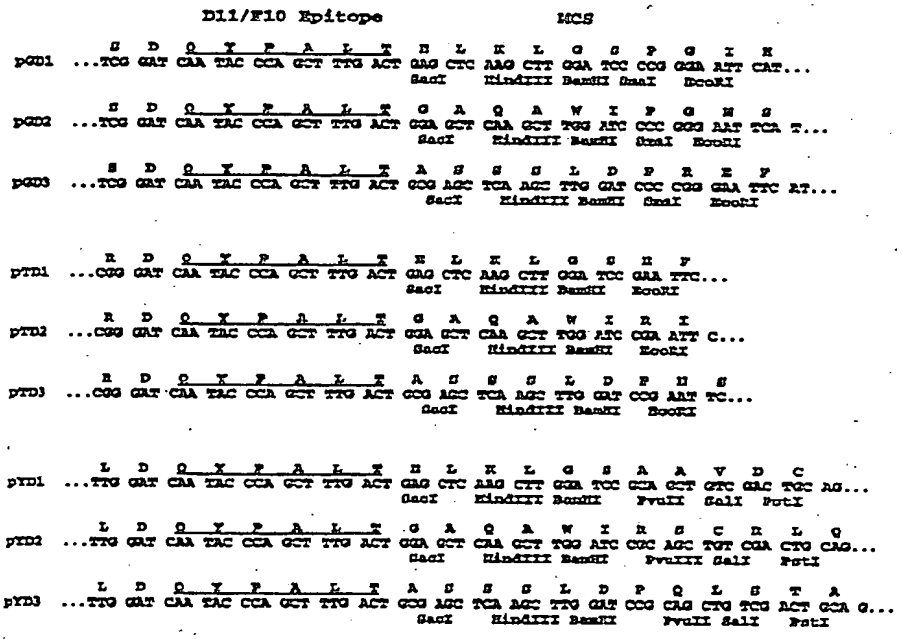

(57) Abstract

Hexapeptides which function as epitope tags when linked to molecules of interest are described. Epitope tags such as those having the sequence Q-Y, F, H or W-P-A, S or V - L or V - T, L, V or Q are useful for purifying a protein of interest, or for studying and characterising a molecule of interest. Preferably QYPALT, QYPSLL, QYPSLQ, QFPALL, QYPVLV and QYPSLT. Most preferably QYPALT.

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EPITOPE TAGGING SYSTEM

Technical Field

This invention is concerned with an epitope tagging system and is particularly concerned with a peptide suitable for use in epitope tagging, an epitope tagged molecule, a hybrid polypeptide containing the epitope tag, an expression vector coding for the hybrid polypeptide, and a process of purifying a protein of interest. The invention also extends to antibodies specific for the peptide tag of the invention.

Background of the Invention

Epitope tagging, also known as immunotagging, epitope flagging, or peptide tagging, is the process of linking a set of amino acid residues that are recognised as an antigenic determinant with a protein of interest (U.S. Patent No. 4782137; Kolodzie and Young, 1991; Field *et al.*, 1988; Pati, 1992; Soldati and Perriard, 1991; Geli *et al.*, 1988; Yee *et al.*, 1987; and Lindsley and Wang, 1993). Tagging a protein with an epitope allows surveillance of the protein by a specific antibody, preferably a monoclonal antibody. This approach can elucidate the size of a tagged protein as well as its abundance, cellular location, posttranslational modification and interactions with other proteins, etc. In particular, epitope tagging allows the protein to be purified even when there is no method of assaying its function.

The epitope tagging approach offers significant advantages over the use of antibodies generated directly against the protein of interest in that:

1) it saves the time and resources which would be expended in making monoclonal antibodies (mAbs);
2) the tagged protein can be monitored with a well-characterised mAb whose spectrum of cross-reactivity with non-tagged proteins is already known;

3) a comparable negative control can be used, which is not possible with independently raised antibodies;

4) the location of epitope in the molecule of interest can be precisely controlled in epitope tagging, which can be very important for structure-function studies; and

5) epitope tagging may be particularly useful for discriminating between similar gene products.

In summary, the epitope tagging approach offers advantages of universality, precision and economy over the use of antibodies raised directly against a protein of interest.

The major uncertainties in an epitope-tagging strategy are:

1) the ability of the tagged protein to maintain its biological function, and

2) the influence of target protein sequence on the antigenicity of the epitope.

For these reasons, it is sometimes essential to develop epitope tags of short length and different sequence characteristics (e.g., different net charges, hydrophobicity and side groups) to increase the chance of success in tagging applications.

To date there are only four tagging systems commercially available, although a number of others have also been described. Two of the systems contain an epitope tag of more than 10 amino acid residues. The only commercial system with an epitope of less than 10 amino acids residues is the FLAG™ system developed by Hopp *et al.* (1988; Prickett *et al.*, 1989). Although FLAG is a small tag (8 amino acid residues), its highly charged nature may prevent its application in tagging protein molecules which are sensitive to net changes in charge.

Table 1 summaries the epitope tag sequences which have been described in the literature, and compares them to the preferred sequence QYPALT of the present invention.

Table 1

Name	Size (aa)	Sequence	Charge	Cognate mAb	Company/Distributor
BTag	6	QYPALT	0	2 (D11,F10)	present invention
FLAG	8	DYKDDDDK	+2/-5	2 (M1,M2)	Immunex/Integrated Sci.; U.S. Patent No. 4782137; U.S. Patent No. 4703004
T7-Tag	21	MASMTGGQQMGR	+1	1	Novagen
HSV-Tag	11	QPELAPEDPED	-5	1	Novagen
VSV-Tag	11	YTDIEMNRLGK	+3/-2	1 (P5D4)	Ref. 1
HA1-Tag	9	YPYDVPDYA	-2	1 (12CA5)	Ref. 2, 3, 4
c-myc	10	EQKLISEEDL	+1/-4	1 (9E10)	Ref. 4, 5, 6
Pk-tag	14	GKPIPNPLLGLDST	+1/-1	1 (SV5-P-k)	Ref. 7, 8
E-tag	13	GAPVPYPDPLEPR	+1/-2	1	Pharmacia

References
1. Soldati and Perriard; Cell, 1991 66 277-289
2. Field *et al*; Mol. Cell. Biol., 1988 8 2159-2165
3. Pati; Gene, 1992 114 285-288
4. Kolodziejj and Young; Meth. Enzymol., 1991 194 508-519
5. Lindsley and Wang; Pro. Natl. Acad. Sci. USA, 1991 88 10485-10489
6. Lindsley and Wang; Nature, 1993 361 749-750
7. Randall *et al*; Vaccine, 1993 11 1247-1252
8. Hanke *et al*; J. Gen. Virol., 1992 73 653-660

The present invention seeks to provide an alternative to the currently available tagging systems.

VP7, a protein of 349 amino acids, is a major structural protein of bluetongue virus (BTV). Although VP7 is recognised by BTV-specific antibodies in group-specific serological tests, it cross-reacts with antibodies to African horse sickness virus and to epizootic haemorraghic disease virus, and thus strictly speaking is not a serogroup-specific antigen.

We have found that a hexapeptide having the sequence Gln-Tyr-Pro-Ala-Leu-Thr (QYPALT), and related peptides derived from the VP7 molecule of BTV, is recognised as an antigenic epitope by two mAbs and can be used as a tag peptide without substantially influencing the antigenicity of the hexapeptide in the tagged molecule. We have designated this sequence BTag.

Summary of the Invention

In a first aspect, the present invention provides a peptide which functions as an epitope tag when linked to a molecule of interest, said peptide having the sequence Q - Y, F, H OR W - P - A, S or V - L or V - T, L, V or Q. More preferably the peptide has a sequence selected from the group consisting of QYPALT, QYPSLL, QYPSLQ, QFPALL, QYPVLV and QYPSLT. Most preferably the peptide has the sequence QYPALT. Without wishing to be limited by any proposed mechanism for the observed beneficial effect, we believe that the amino acids at position 1 and 3 are critical to the peptide, and that the amino acid at position 2 should be aromatic.

In a second aspect, the present invention provides an epitope tagged molecule wherein the epitope tag is, or includes, a hexapeptide of the invention.

Preferably the tagged molecule is a protein, although the invention may encompass any other molecules capable of being linked to the hexapeptide of the invention. The protein may for example be an enzyme,

hormone, genetically engineered single chain antibody molecules which lack an F_c region, or any other protein. More preferably the protein is a recombinant protein. For some purposes the epitope may be linked to a carbohydrate.

The tag may be linked to the molecule of interest by any convenient means. For example, a polypeptide may be synthesized chemically, using conventional solid-phase methods, and the peptide tag incorporated into the synthesis. The tagged polypeptide or protein may be synthesised as a fusion protein by conventional recombinant methods, as described herein. The tag may be coupled to a protein or carbohydrate using conventional cross-linking agents, such as carbodiimide, or using enzymic methods.

The tagged molecule of the invention allows for surveillance of the molecule by a specific antibody as described above. Preferably the antibody is a monoclonal antibody.

In a third aspect, the invention provides a hybrid polypeptide comprising a hexapeptide in accordance with the invention, a protein of interest and one or more linking sequences of amino acids interposed between said hexapeptide and said protein of interest , said linking sequence(s) being cleavable at a specific amino acid by a proteolytic agent.

The proteolytic agent may be an enzyme, such as enterokinase or a chemical agent such as cyanogen bromide. A variety of suitable cleaving agents is known in the art. See for example U.S. Patent No. 4782137; Hopp *et al*, 1988 and Walker *et al*, 1994.

The invention, in a fourth aspect, provides a DNA expression vector comprising DNA coding for a hybrid polypeptide comprising

1) a hexapeptide of the invention,
2) a polypeptide of interest; and optionally
3) at least one linking sequence of amino acids interposed between said hexapeptide and said protein of interest, said linking

sequence being cleavable at a specific amino acid by a proteolytic agent.

The invention also provides a DNA expression vector, comprising DNA coding for a hybrid polypeptide comprising:

1) a hexapeptide according to the invention, and optionally

2) at least one linking sequence of amino acids, said linking sequence being cleavable at a specific amino acid by a proteolytic agent.

A DNA sequence encoding a desired polypeptide or protein can be inserted into the DNA of the vector, so that the hexapeptide and, if desired, the linking sequence, can then be co-expressed with a polypeptide of interest. If the linking sequence is used, the linking sequence is interposed between the hexapeptide and the protein of interest, and can readily be cleaved.

The hexapeptide tag in accordance with the invention may be located at any site in the desired protein, ie. the N-terminus, at any site within the sequence, or at the c-terminus of the hybrid polypeptide. For specific purposes the N-terminus or C-terminus may be particularly convenient.

In yet a further aspect, the invention provides a method for producing a hybrid polypeptide in accordance with the second aspect by transforming host cells with the DNA expression vector of the third aspect and expressing said hybrid polypeptide.

The host cell may be anu suitable cell type, such as a bacterium, a eukaryotic cell such as a yeast, or a mammalian cell.

In addition, the epitope tag of the invention is suitable for use in phage display expression systems. Thus, the epitope tag of the invention may be used to tag a structural component of a recombinant virus.

The invention also provides an expression vector comprising sequences coding for the hexapeptide of the invention and having one or more cloning sites. The vector may have multiple cloning site in three reading frames.

In still a further aspect, the present invention provides a method for purifying or isolating a protein of interest comprising subjecting the hybrid polypeptide of the invention to affinity chromatography. The affinity separation may, for example, be achieved by contacting the hybrid polypeptide with an immobilised antibody, especially a monoclonal antibody, specific for the hexapeptide tag of the invention. A variety of coupling methods and solid supports for the immobilisation of antibodies to form a support suitable for affinity chromatography are known. A person skilled in the art will be able to select an appropriate system readily.. The purified protein may then be cleaved from the peptide tag.

In a further aspect, the invention provides antibodies, including monoclonal antibodies, capable of recognising the hexapeptide tag of the present invention. Methods for production and screening of polyclonal or monoclonal antibodies are well known in the art. Particularly preferred are monoclonal antibodies designated F10 and D11 as described hereunder. The invention extends to hybridoma cells capable of producing monoclonal antibodies in accordance with the invention, and to assay systems, such as competition ELISA assays utilising a recombinant antigen in which the epitope of the invention is located adjacent to the immunogenic region of a target protein.

It is envisaged that the monoclonal antibodies of this invention can be applied not only to purification and assay of recombinant or other proteins tagged with the peptide of the invention, but also to detection of a variety of other types of compounds thus tagged. For example, environmental monitoring of effluent streams can be achieved by tagging a component of the effluent or by

adding a tagged marker to the effluent, and assaying environmental samples for the presence of the tag.

The antibodies of the invention may be conjugated to an enzyme or other signal system commonly used in the art, for example, horseradish peroxidase, for use in the detection methods described above.

In another aspect, the invention relates to a kit comprising an epitope tag in accordance with the invention, and an antibody to the tag or hexapeptide of the invention.

In a further aspect, the invention provides a kit comprising a plurality of epitope-tagged molecules, wherein the tagged molecules are a series of molecular weight standards or markers.. Preferably, the epitope-tag is a hexapeptide having the sequence QYPALT. Such a kit would be useful in Western blot detection of purified proteins to estimate protein size.

Detailed Description of the Invention

The invention will hereafter be described in detail with reference to the following non-limiting examples. In this more detailed description of the invention reference will be made to the accompanying drawings in which:

Figure 1 shows deletion mapping in the pGEX vector. The black arrowed bar on top of the figure represents the coding region for BTV VP7 with important restriction sites marked above. abbreviations for restriction enzymes are: B, BamHI; Bg, Bg1II; Bs, BsmI; E, EcoRI; H, HindIII; N, NdeI; Na, NaeI; R, RsaI; S, Sau3A; parentheses indicate that the restriction sites were not present in the original gene sequence and were generated by PCT mutagenesis. Three important residues (Cys-15, Cys-65 and Lys-255) are indicated by up-pointing open arrows. The dotted bars in the centre represent the gene fragments is given at the bottom in base pairs (bp). Plasmid names are given at the left, while the corresponding amino acid numbers (AA#) for each insert in these plasmids are given

at the right. The reactivity of each recombinant fusion protein to D11 and F10 is indicated by "+" (reactive) and "-" (non-reactive).

Figure 2 shows results of SDS-PAGE and Western blot analysis of GST-fusion proteins. Panel A is a Coomassie Blue stained gel while panel B is a Western blot. Lane 1: GST derived from control vector pGEX-1N; Lanes 2-4: GST-fusion proteins derived from expression plasmids pGEX-BR, pGEX-BB, and pGEX-BS, respectively (see Figure 1 above).

Figure 3 shows results of ELISA analysis of mAb binding to overlapping synthetic peptides of various lengths. The results obtained for D11 are presented in the left four panels, while those for F10 are shown on the right. The length of peptides is indicated at the upper right corner of each panel. The numbers on the Y-axis represent OD readings, while the residue numbers 1 to 20 given on the X-axis correspond to residues 256 to 275 in the BTV-1 VP7 molecule (Eaton *et al.*, 1991).

Figure 4 provides sequences for nine expression vectors, indicating the tagged QYPALT epitope and the multiple cloning sites in three reading frames.

Figure 5 shows results of Western blot analysis of GST-tag proteins from pGD vectors. Panel A is a Coomassie Blue stained gel while panels B and C are Western blots with D11 and F10, respectively. Lane 0: GST expressed from control vector pGEX-1N; lanes 1-3: GST-tag proteins derived from pGD1, pGD2 and pGD3, respectively (see Figure 4 for sequences).

Figure 6 shows a Western blot of recombinant VP7 proteins from a previously unknown serotype, designated BTV-0, and from BTV-15. Panels A and B are Western blots probed with D11 and F10, respectively. Lane 1: GST-fusion protein derived from pGEX-BS (see Figure 1 and Figure 2) used as positive control; lane 2: BTV-0 VP7 expressed from pET vector (Studier *et al.*, 1990); lane 3: BTV-15 VP7 expressed from the same pET system.

Figure 7 shows a comparison of deduced amino acid sequences for VP7 molecules of BTV-1, BTV-0 and BTV-15. Identical amino acid residues are indicated by dots, while those different from BTV-1 are given in one-letter amino acid codes.

Figure 8 illustrates the mechanism of a competition ELISA assay.

Panel A shows competition between antibodies binding to the same epitope.

Panel B shows competition between antibodies binding to neighbouring epitopes.

Example 1 Identification of Epitope of Bluetongue Virus VP7 Molecule Recognised by two Monoclonal Antibodies

For structure and functional studies of BTV viral proteins, a panel of mAbs was generated against the major core protein VP7.

Mice were immunised with Australian BTV serotype 1 which had been denatured with sodium dodecyl sulphate, and hybridoma cells were established and maintained as ascites cells in mice using conventional methods, as generally described by Lunt *et al* (1988). Spleen cells from immunized Balb/c mice were fused with cells of mouse myeloma cell line Ag14-Sp2/0 (Schulman *et al*, 1978), using polyethylene glycol MW1500 (BDH) as fusion agent.

Hybridoma cell lines were screened by indirect ELISA using BTV antigens which had been partially purified by centrifugation through 40% sucrose. Subclones were generated from positive clones by limiting dilution of the parent lines, and screened by ELISA and Western blotting using recombinant VP7 produced in yeast in order to identify positive subclones. Monoclonal antibodies were purified from mouse ascitic fluid using Protein A affinity chromatography.

Among these mAbs, two particular mAbs (D11 and F10) showed strong binding activity in Western blot

analysis, indicating that they recognise a linear epitope on the VP7 molecule.

Using recombinant peptides expressed as fusion proteins with glutathione-S-transferase (GST) in the pGEX vectors (Smith and Johnson, 1988), it was shown that both D11 and F10 bind to a 20 amino acid (aa) peptide between residues 256 and 275 in the VP7 molecule. This is illustrated in Figures 1 and 2. Further characterisation of the molecular structure of these mAb-defined epitopes was carried out using systematic scanning of overlapping synthetic peptides by the PepScan method of Geysen *et al* (1984) and by random epitope library methods (Scott *et al*, 1990; Devlin *et al*, 1990; Cwirla *et al*, 1990).

These techniques allowed an epitope consisting of 6 contiguous amino acid residues to be precisely delineated. In addition, the epitope library made it possible to identify some of the residues important for recognition, as well as to predict the presence of a potentially cross-reactive determinant on EHDV.

For the PepScan analysis, hybridoma cell culture supernatants of D11 and F10 by ELISA assays were used at 1:20 dilution. Overlapping peptides (5- to 8-mers) were synthesized and tested for mAb binding. The results, presented in Figure 3, show that although both antibodies recognised the penta-peptide QYPAL located at amino acid positions 259-263, the hexapeptide QYPALT produced higher signals. mAb F10 also reacted with TAEIFNV, and adjacent heptapeptide, suggesting that it may recognize a discontinuous epitope incorporating contact sites on both peptides, perhaps, juxtaposed by a potential β-turn (Chou and Fasman, 1974). PepScan analysis thus showed that both mAbs recognize the same region of VP7, but indicated that they may have slightly different specificities. Thus the main antigenic determinant for both mAbs was a 6-aa peptide with the sequence QYPALT.

Example 2 Identification of Variant Sequences Recognised by MAbs

Selection of peptides from a filamentous phage library displaying random hexapeptides (Scott and Smith, 1990) was used as an adjunct to PepScan for fine epitope mapping. Affinity purification of binding phages was essentially as described by Scott and Smith (1990) and by Parmley and Smith (1988), and was carried out by subjecting the library to three rounds of biopanning and one of micropanning with each of the MAbs. DNA of the affinity-selected phages was sequenced using the fUSE sequencing primer (Parmley and Smith, 1988) and the Taq Track sequencing system (Promega) as described by the manufacturer.

After the three successive rounds of biopanning, phages produced by 93 separate tetracycline-resistant transductant bacterial colonies were picked for micro-panning to confirm that their displayed hexapeptides were individually recognised by the respective antibodies. Only micropanned colonies that yielded more than 100 transductant colonies per 20 µl spot were picked for DNA sequencing. Comparison of their deduced amino acid sequences showed that MAb D11 had selected phages displaying three very similar hexapeptides, as shown in Table 2.

Table 2

Phage Displaying Hexapeptides Selected From The Epitope Library By MAbS D11 and F10

MAb D11	MAb F10
QFPALL (14)	**QFPALL** (17)
QYPSLL (6)	**QYPSLL** (1)
QYPVLV (1)	**QWPAVL** (1)

The number of phage clones displaying each of the hexapeptide classes is shown in brackets. Residues similar or identical to the authentic VP7 sequence (QYPALT) at positions 259 to 264 are shown in bold

Inspection of the amino acid sequence of VP7 of BTV revealed that these hexapeptide most closely approximated the residues QYPAL(T) located at positions 259-264 in 8 of the BTV VP7 sequences so far characterized. We have found that two isolates differ in this region; BTV 0 has the sequence QYPSLT and BTV-15 has QYPALA. MAb F10 also selected very similar peptides from the library. None was exactly identical to QYPALT, perhaps since many hexapeptides may be missing from such a library simply by chance (Scott and Smith, 1990). Nevertheless, sequences of the peptides isolated from the epitope library correlated well with those recognised by the PepScan method. MAb F10, however, also bound the heptapeptide TAEINFV in PepScan (Figure 3), while none of the affinity-purified phages expressed comparable sequences. A possible reason for this apparent discrepancy is that if the library contained only distantly related hexapeptides, they may have had a low affinity for the paratopes and were thus eliminated during biopanning. Furthermore, the way in which PepScan and phage display peptides are presented is probably not strictly comparable. Nevertheless, both techniques identified QYPALT, an epitope that is distinct from the three antigenic regions on VP7 that have previously been identified (Li and Yang, 1990; Eaton *et al*, 1991).

To confirm that their displayed peptides represented a BTV epitope, phages expressing each of the affinity selected sequences were propagated, purified as described by Scott and Smith (1990), and tested by ELISA with the two MAbs and a polyclonal antiserum raised against BTV in a rabbit. The results, presented in Table 3, show that MAb F10 reacted with all four biopanned peptides, but D11 did not recognize QWPAVL, a sequence that had been isolated only by MAb F10.

Table 3

ELISA Analysis of Representative Recombinant Phages

Sequence	ELISA $A_{450\ nm}$ with D11	F10	BTV4 As
QFPALL	0.646	0.866	0.392
QYPSLL	0.395	0.467	0.261
QYPVLV	0.525	0.842	0.229
QWPAVL	0.066	0.733	0.128
11 Phage	0.005	0.000	0.053

ELISA plate wells were coated with phage preparations adjusted to an A_{288} of 0.25. Monoclonal antibodies were added at a concentration of 10 µg/ml and the rabbit antiserum at a dilution of 1/100. MAbs were detected with peroxidase-conjugated rabbit antimouse IgG (Dakopatts) and rabbit antibodies were peroxidase-conjugated protein A (Cappel). Results represent averages of three determinations.

The epitope library therefore confirmed that the fine specificities of the two mAbs were different, an observation further supported by our finding that neither D11 nor F10 binds VP7 of BTV-15 while F10, but not D11, binds VP7 of BTV-0. The rabbit antiserum which was directed against isolate BTV4 also recognised all the selected peptides, indicating that the QYPALT region was antigenic in the purified virus preparation used as immunogen.

In addition to precise epitope mapping, comparison of the affinity-purified peptide sequences allows residues critical to the antibody-antigen interaction to be identified (Scott, 1992). For the BTV-specific MAbs F10 and D11, Q and P are probably critical, an aromatic residue in position 2 is obviously important, while position 5 is preferentially occupied by the related

L or V. The L (or V in one clone panned by D11) at position 6 also seems to be important in the phage system (Table 3), which was interesting since in VP7 this position is occupied by T. Position 4 may not represent a critical contact residue for either antibody since it can accommodate an A, V (both aliphatic, non-polar), or S (polar, hydroxyl). The phage display system does not, however, accurately reflect the situation in the viral protein. For example, both MAbs fail to bind VP7 of BTV-15 which has QYPALA in the relevant region. Despite none of the panned phages displaying a T at position 6, the change from T to A in the sixth residue of the authentic epitope is clearly crucial to recognition. This finding implies that although the random epitope library can identify some critical residues in a given epitope, its ability may be limited, perhaps due to the unpredictable effects of flanking sequences.

Example 3 Confirmation that the hexapeptide QYPALT is the principal determinant for the binding of both D11 and F10

To verify that the hexapeptide QYPALT is the principal determinant for the binding of mAbs D11 and F10, synthetic oligonucleotide primers were made in order to introduce the 6-aa epitope tag into different expression vectors and to test its antigenicity in tagged recombinant proteins. This was achieved using oligonucleotide primers and polymerase chain reaction (PCR; Saiki *et al*, 1985). Small PCR fragments encoding the epitope were inserted into expression vectors. The three classes of epitope tagging vectors used in this example, designated pD, pTD and pYD, were derived from three widely used *E. coli* and yeast expression vectors: pGD was derived from pGEX-1N (Smith and Johnson, 1988), pTD was derived from pET-5b (Studier *et al.*, 1990) and PYD was derived from pYELC5 (Macreadie *et al.*, 1989; Macreadie 1990). All of these original vectors contain a unique BamHI cloning site.

The construction process was same for each of the three classes. To insert the coding sequence for QYPALT upstream of multiple cloning sites, three small DNA fragments were synthesised. Each of the three fragments differs by a single base pair at the 3'-end of the QYPALT coding region, so that each of them will generate a different reading frame for fusion of foreign genes. Insertion of these three small gene cassettes into the unique BamHI site of each of the three original expression vectors resulted in the nine new vectors, whose sequences are shown in Figure 4.

For each class of the three vectors (e.g., pGD1, 2, 3), fusion can be made using any one of the three possible reading frames for any particular restriction site present in the multiple cloning site.

The antigenicity of the tagged GST proteins expressed from the pGEX-derived vectors, pGD1, pGD2 and pGD3, was tested by Western blot. The results, presented in Figure 5, indicated that all three GST-tag proteins maintained the antigenicity of the tag. However, in the case of pGD1 the antigenicity seemed to be weakened, probably due to the presence of a highly positively charged Lys residue downstream of the epitope sequence (see Figure 4). This indicates that the antigenicity of a given epitope tag can be affected by the sequence environment of the target protein. The results also demonstrated that the weakening effect in pGD1 was less severe for F10 than for D11.

Example 4 Application of the tagging system in monitoring and purification of recombinant proteins

Having verified that the antigenicity of the epitope tag in pGD vectors is retained, various tagged proteins were expressed in the bacteria *Escherichia coli* and *Bacillus subtilis* and in the yeast *Saccharomyces cerevisiae*, and in baby hamster kidney (BHK) cells.

We have now expressed 23 different proteins using the epitope tag of the invention. In all of the experiments, we were able to generate a functional epitope tag which could be detected by immunological methods, such as Western blotting, ELISA, immunoprecipitation, or immunofluorescent microscopy. No cross-reactive protein bands have been detected in any of the host systems tested. Moreover, a sequence homology search of public sequence data banks, including GenBank, revealed no protein including the sequence QYPALT.

We have observed no adverse affects on either the expression or the function of the tagged recombinant proteins, including complex functions such as protein secretion, membrane insertion, and virus assembly.

We have been able to utilise the epitope tag of the invention in a variety of expression systems and recombinant hosts, including *Escherichia coli* (using both plasmid and phage systems), *Bacillus subtilis*, the yeast *Saccharomyces cerevisiae*, and mammalian cell lines such as BHK cells. We consider that the epitope tag of the invention is generally applicable, and can also be used in a system such as baculovirus.

In the tagging experiments which we have performed, the epitope tag has been placed at the N-terminus, at the C-terminus, or in the interior of the sequence of the recombinant protein. Our results indicate that the epitope tag of the invention is a strong antigenic determinant, which can be utilised adjacent to a variety of flanking sequence environments without significant loss of antigenicity.

Example 5 C-terminal tagging

As shown in Figure 4, BTag was initially incorporated into three different expression systems (the pGEX vector, the T7 RNA polymerase based pET vector and pYELC, a yeast expression vector) in all three reading frames, thus resulting in nine different fusion vectors.

In the case of the pGD vectors, the BTag was placed at the C-terminus of the GST protein in the pGEX vector, so-called "C-terminal tagging". The antigenicity of BTag in all three vectors has been tested and confirmed by Western blotting.

When probed with mAb D11, the antigenicity of BTag in pGD1 was weaker compared with that in pGD2 and pGD3, probably due to the presence of the Glu(E) and Lys(K) residues immediately downstream (see Figure 5). However, when mAb 20F10 was used in a Western blot, the difference was less of a problem. The decrease in antigenicity of BTag in pGD1 may be overcome by insertion of the GCG (Ala) codon between the BTag and the Glu(E) codon to introduce the small Ala residue, which was present in the native sequence of BTV VP7 protein, and to maintain the reading frame.

In addition to these examples of GST-BTag expression in pGD vector, we have also expressed a C-terminal tagged scFv antibody molecule in *E. coli*. In this experiment, the peptide of the invention was found to be superior to other tagging systems which had been previously used for expression of scFv, such as FLAG, in that the antigenicity was stable and no adverse effects were observed on the expression of the recombinant scFv molecules.

The C-terminal tagging experiments are summarized in Table 4.

Table 4

Protein	Expression vector	Expression host
GST	pGD	*E. coli*
GST	pGD	*E. coli*
GST	pGD	*E. coli*
scFv	pPOW	*E. coli*

pPOW is an *E. coli* vector for expression of single chain antibodies (Power *et al*; Gene, 1992 113 95-99).

Example 6 N-terminal tagging

Two groups of N-terminal fusions have been conducted. The first group was obtained by cloning of target gene (fragments) into pTD or pYD vectors. In these cases, BTag was not placed precisely at the N-terminus, but was located at a position 13 and 6 amino acids respectively from the N-terminus. The second group of N-terminal tagging proteins was obtained by insertion of small double stranded DNA fragments generated by PCR of overlapping primers, which code for the BTag sequence at the very beginning of the protein coding regions, thus forming a genuine "N-terminal tag". Five fusion proteins were expressed in the first group (three from pTD vectors and two from pYD vectors). All of them were viral surface proteins.

In the second group, the BTag was placed at the N-terminus of filamentous phage fd coat proteins pIII and pVIII. Four different fusion proteins have been constructed, two for pIII fusion and two for pVIII fusion. In these cases, the BTag was placed immediately after the signal peptide cleavage site. The functional incorporation of BTag into phage particles indicates that the BTagged fusion coat proteins were secreted, processed and assembled as for normal wild-type protein.

The results are summarised in Table 5.

Table 5

Protein	Expression vector	Expression host
Classical swine fever virus (CSFV)-gp55	pTD	*E. coli*
CSFV-gp55	pTD	*E. coli*
CSFV-gp55	pYD	yeast
African swine fever virus (ASFV)-p72	pTD	*E. coli*
AFSV-p72	pYD	yeast
rabbit calicivirus VP-60	pTD	*E. coli*
fd phage pIII	fd-tet	*E. coli*
fd phage pIII	fd-tet	*E. coli*
fd phage pVIII	fd-tet	*E. coli*
fd phage pVIII	fd-tet	*E. coli*

fd-tet is a class of vectors derived from the filamentous phage fd (Parmley and Smith, 1988).

Example 7 Internal Tagging

Internal tagging can also be divided into two groups. The first involved the expression of recombinant proteins in pGD vectors, while the second group was obtained by insertion of the BTag coding fragment.

Three viral structural proteins have been expressed in pGD vectors, forming GST-BTag-target protein fusions.

In the second group, three different fusion proteins have been expressed. Two of them were expressed in *Bacillus subtilis*, with the BTag being inserted into the pro-peptide region of an extracellular protein. The function of the fusion protein was fully maintained,

indicating that BTag had no effect on protein secretion and processing. The recipient protein in the third fusion was a viral membrane glycoprotein and in this instance, BTag was inserted into a small domain predicted to be exposed on the cell surface. The fusion construct was expressed in recombinant vaccinia virus, and the BTag was detected by immunofluorescent microscopy on the infected cell surface. The results are summarised in Table 6.

Table 6

Protein	Expression vector	Expression host
CSFV-gp55	pGD	*E. coli*
bovine viral diarrhoea virus - gp53	pGD	*E. coli*
BTV-15 VP7	pGD	*E. coli*
neutral protease	pNC3	*Bacillus subtilis*
neutral protease	pNC3	*B. subtilis*
neutral protease	pNC3	*B. subtilis*
BTV-1 NS3	vaccinia virus	BHK cells
RCV - VP60	pGD	*E. coli*

pNC3 is a *B. subtilis* expression vector comprising the gene for extracellular neutral protease (Wu *et al*; Gene, 1991 106 103-107).

Example 8 Effect of Sequence Variations on Antigenicity

As mentioned previously, the antigenicity of the epitope tag was somewhat weakened in pGD1 (Figure 5), but this effect was less severe with mAb F10 than D11 (compare lanes 1 in Figure 5B and 5C respectively). Further evidence comes from Western blot analysis of BTV VP7

molecules from two different serotypes, results of which are presented in Figure 6. While the single point mutation in the epitope region of BTV-0 VP7 (changing from QYPALT to QYPSLT, see Figure 7 for sequence comparison) eliminated the binding of D11, F10 was still able to bind, albeit with a decreased efficiency.

Example 9 Affinity Chromatography Purification of Epitope-Tagged Protein

An affinity column was prepared using MAb D11 and Affi-Gel resin (Bio-Rad) using the procedure used by the manufacturer, and equilibrated with phosphate-buffered saline, pH 7.2. The recombinant protein was BTV-1 VP7 from yeast, which carries the QYPALT tag within the sequence.

A crude total cell lysate was prepared by sonication and centrifugation.

The crude cell lysate was prepared in phosphate buffered saline, the buffer used to equilibrate the column. The lysate was applied through the column twice.

The column was eluted with 2x bed volumes of 0.5 formic acid, pH approximately 2.5. The eluate was collected in 8 fractions and neutralised with NaOH.

The fractions were assayed by three different methods:

1) OD_{280};
2) ELISA using mAb D11 and a 1:400 dilution of the elute;
3) Western blot using mAb D11 and 5µl eluate.

The results are summarised in Table 7.

Table 7

Fraction #	OD_{280}	ELISA reading (1:400)	Western Blot
1	0.465	0.037	ND
2	0.615	0.021	ND
3	0.600	0.025	-
4	0.603	0.110	++
5	0.630	0.096	++
6	0.539	0.083	++
7	0.242	0.072	+
8	0.119	0.000	-

Although in this preliminary experiment no quality control was carried out during he preparation of the column, and the ratio of immobilised D11 to recombinant antigen was not optimised, the results indicated that D11 was able to bind recombinant BTV-VP7 under the conditions used, and that the recombinant protein could be eluted with formic acid.

Example 10 Competition ELISA Assay Using Monoclonal Antibodies of the Invention

Antibody detection is one of the most convenient methods for diagnosing or monitoring viral or bacterial infection in humans and animals. With the advance of recombinant DNA technology, recombinant antigens are nowadays readily available for such purposes. However, serum antibodies can cause a problem of high background when assayed directly. As shown in Figure 8, competition-ELISA (C-ELISA) is based on the fact that the binding of mAb to a specific antigen can be blocked or reversed by the competitive binding of serum antibodies to the same epitope

(Figure 8A) or an epitope in close proximity (Figure 8B).

The main difficulty in devising an effective C-ELISA is the time-consuming process of producing and screening for a suitable mAb for such application.

We consider that it should be possible to device C-ELISA by engineering a recombinant antigen with the small BTag placed next to an immunogenic region of any target protein. In this case, the C-ELISA will mainly utilise the mechanism outlined in Figure 8B, ie. competition between the binding of mAb to BTag and the binding of polyclonal antibodies to nearby epitopes on the target antigen.

Two specific examples have been tried:

a) The first example used the recombinant protein CSFV-gp55, which has the following configuration:

N--T7 tag::BTag::CSFV-gp55(C-terminal 1/3 of molecule)--C

When an ELISA assay was performed using mAb D11, approximately 60-70% inhibition was caused by rabbit anti-T7 tag antibodies (purchased from Novagen). This was significant, considering that control rabbit serum gave no inhibition and that this was a "heterologous" competition system in which the mAb directed epitope was unrelated to the target epitope being assayed (in this case the T7 tag epitope).

b) We have also used another recombinant protein, a GST-fusion protein for RCV-VP60 with the following configuration:

N--GST::BTag::RCV-VP60--C

When assayed with mAb D11 in competition with sheep anti-RCV polyclonal antibodies, an average inhibition of 75% was observed compared with control sheep serum.

We conclude that these results suggest that the competition had been achieved, and that due to its heterologous nature the inhibition may never reach 100% as in the case of homologous C-ELISA.

One of the unique features of the epitope tagging system of the present invention is that the tag peptide is recognised by two individual mAbs, yet still maintains its

high specificity. This is very useful in certain applications when different binding affinities are required, e.g. in competition-ELISA analysis where excessive binding of the mAb may prevent competition for the epitope by antibodies in test sera. Furthermore, sequence variations flanking or within the epitope tag may affect mAb binding. Having two mAbs in the tagging system increases the chance of success in tagging applications where the failure of binding by one mAb may be offset by the success of the other.

Example 11 Affinity Columns

Affinity purification columns were prepared using each of D11 and F10 monoclonal antibodies coupled to cyanogen bromide-activated Sepharose resin (Pharmacia) according to the manufacturers' protocols. The optimal coupling level for each antibody was determined to be 5 mg/ml of resin.

Table 8 shows *E. coli* recombinant proteins containing the epitope tag QYPALT which were generated.

Table 8

Protein	Description	Vector	Protein size	QYPALT Location
GST	Glutathione S Transferase	PGD3	26 KD	C Terminal (to GST)
GST-PBC	Primary Billiary Cirrhosis, Autoantigen - GST Fusion Protein	pGEX-4T	74 KD	Amino Acids (between GST and PBC sequence)
La[1]	La Autoantigen	PQE30	45 KD	Amino Acids 344-361

Crude cell lysates were prepared for each of the above proteins by sonication and centrifugation.

500 µl of each lysate was incubated with 500 µl of D11 coupled resin equilibrated with 100 mm phosphate buffer, pH 7.0. In addition, for GST 500 µl of lysate was

incubated with 500 µl of F10 coupled affinity resin. Lysates and resins were incubated with gentle agitation for 30 minutes at room temperature to allow binding. Incubation beyond 30 minutes has been shown to result in reduced yield due to irreversible binding.

Elution was achieved via use of 100 mM glycine pH 3.5. pH values greater than 3.5 result in reduction in protein yield.

Elution fractions were analysed for purity, immunoreactivity and yield by the following methods:

1. OD280
2. Bradford protein estimation
3. Coomassie stained PAGE.
4. Western blot (using mABs D11 and F10 conjugated to horseradish peroxidase).

The results obtained are shown in Table 9.

Table 9

PROTEIN	D11 AFFINITY COLUMN PURIFICATION			F10 AFFINITY COLUMN PURIFICATION		
	Total Yield[1]	Purity[2]	Western Blot Result	Total Yield	Purity	Western Blot Result
GST	270 µg	>95%	++++	300 µg	>95%	++++
PBC-GST	686 µg	50%[3]	+++	NA	NA	NA
La - Centrally Tagged	216 µg	>90%	++	NA	NA	NA

NOTES:

1 Yield obtained is from 500 µl lysate incubated with 500 µl antibody coupled resin and represents a total yield after pooling elution fractions.

2 Purity as determined by Coomassie PAGE. In the case of La - centrally tagged a silver strain gel was utilised

3 PBC-GST shows 50% purity post affinity column with contaminating band almost entirely free GST. The same profile is observed for PBC- GST purified on glutothione sepharose.

Although the invention has been described with reference to the tagging of characterised proteins, the current advance of molecular biology and the ease with which small tag sequences can be inserted into any unknown random cloned gene sequences by the technology of polymerase chain reaction (PCR), the present invention provides an epitope tagging system which is generally applicable.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

References cited herein are listed on the following pages, and are incorporated herein by this reference.

REFERENCES

1. Doldati, T. and Perriard, J-C.
"Intracompartmental sorting of essential myosin light chains: molecular dissection and in vivo monitoring by epitope tagging"
Cell, 1991 66 277-289.

2. Eaton, B.T., Gould, A.R., Hyatt, A.D., Coupar, B.E.H., Martyn, J. C. and White, J.
"A bluetongue serogroup-reactive epitope in the amino-terminal half of the major core protein VP7 is accessible on the surface of bluetongue virus particles"
Virology, 1991 180 687-696.

3. Field, J., Nikawa, J.-I., Broek, D., MacDonald, B., Rodgers, L., Wilson, I.A., Learner, R.A. and Wigler, M.
"Purification of a RAS-responsive adenylyl cyclase complex from *Saccharomyces cerevisiae* by use of an epitope addition method"
Mol. Cell. Biol., 1988 8 2159-2165.

4. Geli, V., Baty, D. and Lazdunski, C.
"Use of a foreign epitope as a "tag" for the localisation of minor proteins within a cell: the case of the immunity protein to colicin A"
Proc. Natl. Acad. Sci. USA, 1988 85 689-693.

5. Hopp, T.P., Prickett, K.S., Price, V.L., Libby, R.T., March, C.J., Cerretti, D.P., Urdal, D.L. and Conlon, P.J.
"A short polypeptide marker sequence useful for recombinant protein identification and purification"
Bio/Technology, 1988 61204-1210.

6. Kolodziej, P.A. and Young, R.A.
"Epitope tagging and protein surveillance"
Meth. Enzymnol, 1991 194 508-519.

7. Lindsley, J.E. and Wang, J.C.
"Study of allosteric communication between promoters by immunotagging"
Nature, 1993 361 749-750.

8. Lunt, R.A., White, J.R. and Blacksell, S.D.
"Evaluation of a monoclonal antibody blocking ELISA for the detection of group-specific antibodies to bluetongue virus in experimental and field sera"
J. Gen. Virol., 1988 69 2729-2740

9. Macreadie, I.G.
"Yeast vectors for cloning and copper-inducible expression of foreign genes"
Nucleic Acids Research, 1990 18 1078.

10. Macreadie, I.G., Jagadish, M.N., Azad, A.A. and Vaughan, P.R.
"Versatile cassettes designed for the copper inducible expression of proteins in yeast"
Plasmid, 1989 21 147-150.

11. Pati, U.K.
"Novel vectors for expression of cNDA encoding epitope-tagged proteins in mammalian cells"
Gene, 1992 114 285-288.

12. Prickett, K.S., Amberg, D.C. and Hopp, T.P.
"A calcium-dependent antibody for identification and purification of recombinant proteins"
BioTechniques, 1989 6 580-589.

13. Schulman *et al*
Nature, 1978 276 269-270

14. Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T. and Arnheim, N.
"Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anaemia"
Science, 1985 230 1350-1354.

15. Smith, D.B. and Johnson, K.S.
"Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase"
Gene, 1988 67 31-40.

16. Studier, F.W., Rosenberg, A.H., Dunn, J.J and Dubendorff, J.W.
"Use of T7 RNA polymerase to direct expression of cloned genes"
Methods in Enzymology, 1990 185 60-89.

17. Yee, J.-K., Jolly,D.J., Miller, A.D., Willis, R., Wolff, J. and Friedman, T.
"Epitope insertion into the human hypoxanthine phophoribosyltransferase protein and detection of the mutant protein by anti-peptide antibody"
Gene, 1987 53 97-104.

18. Li, J.K.-K. and Yang, Y.-Y.
Virology, 1990 178 552-559

19. Geysen, H.M., Meoen, R.H. and Barteling, S.J.
Proc. Natl. Acad. Sci. USA, 1984 81 3998-4002

20. Scott, J.K. and Smith, G.P.
Science, 1990 249 386-390

21. Devlin, J.J., Panganiban, L.C. and Devlin, P.E.
Science, 1990 249 404-406

22. Cwibla, S.E., Peters, E. Barret, R.W. and Dower, W.J.
Proc. Natl. Acad. Sci. USA, 1990, 87 6378-6382

23. Chou, P.Y. and Fasman, G.D.
Biochemistry, 1974 13 222-245

24. Parmley, S.F. and Smith, G.P.
Gene, 1988 73 305-318

25. Walker, P.A., Leong, L.E.-C., Ng, P.W.P., Tan, S.H., Waller, S., Murphy, D. and Porter A.G.
"Effecient and rapid affinity purification of proteins using recombinant fusion proteases"
Bio/Technology, 1994 12 601-605

26. Scott, J.K.
Trends Biochem. Sci., 1992 17 241-245

CLAIMS:

1. A peptide which functions as an epitope tag when linked to a molecule said peptide having the sequence Q - Y, F, H or W - P - A, S or V - L or V - T, L, V or Q.

2. A peptide according to Claim 1, having a sequence selected from the group consisting of QYPALT, QYPSLL, QYPSLQ, QFPALL, QYPVLV and QYPSLT.

3. A peptide according to Claim 2, having the sequence QYPALT.

4. An epitope-tagged molecule, wherein the epitope tag comprises a hexapeptide according to any one of Claims 1 to 3.

5. An epitope-tagged molecule, wherein the epitope tag is a hexapeptide according to any one of Claims 1 to 3.

6. A hybrid polypeptide, comprising a hexapeptide according to any one of Claims 1 to 3, a molecule tagged with said hexapeptide and optionally one or more linking sequences of amino acids interposed between the hexapeptide and the tagged molecule, said linking sequence being cleavable at a specific amino acid by a proteolytic agent.

7. A DNA expression vector, comprising DNA coding for a hybrid polypeptide comprising:

1) a hexapeptide according to any one of Claims 1 to 3,
2) a polypeptide to be tagged with said hexapeptide; and optionally
3) at least one linking sequence of amino acids interposed between said hexapeptide and said polypeptide, said linking sequence being cleavable at a specific amino acid by a proteolytic agent.

8. A DNA expression vector, comprising DNA coding for a hybrid polypeptide comprising:

1) a hexapeptide according to any one of Claims 1 to 3; and optionally
2) at least one linking sequence of amino acids, said linking sequence being

cleavable at a specific amino acid by a proteolytic agent.

9. A method of producing a hybrid polypeptide comprising an epitope-tagged molecule, comprising the steps of transforming a host cell with a DNA expression vector according to Claims 7 or 8, and expressing said hybrid polypeptide.

10. A method according to Claim 9, wherein the host cell is selected from the group consisting of a bacterium, a eukaryotic cell and a mammalian cell.

11. A method according to Claim 10, wherein the host cell is selected from the group consisting of *E. coli*, *B. subtilis*, *S. cerevisiae* and baby hamster kidney cells.

12. A tagged molecule according to any one of Claims 4 to 6, wherein said tagged molecule is a protein selected from the group consisting of an enzyme, hormone, a genetically engineered single chain antibody molecule which lacks an F_c region, and a recombinant protein.

13. A tagged molecule according to any one of Claims 4 to 6, wherein said tagged molecule is a structural component of a recombinant virus.

14. A tagged molecule according to Claims 4 or 5, wherein said tagged molecule is a carbohydrate.

15. A tagged molecule according to Claim 12, wherein the hexapeptide tag is located at the N-terminus of the protein, at any site within the sequence of the protein or at the C-terminus of the protein.

16. A method of purifying or isolating a protein or tagged protein, comprising subjecting a tagged protein according to Claim 12 to affinity chromatography.

17. A method according to Claim 16, wherein the hexapeptide tag is cleaved from the tagged protein following said affinity chromatography.

18. A method according to Claim 16, wherein the tagged protein is contacted with an immobilised antibody specific for the hexapeptide tag, and said protein is purified or isolated by cleaving the hexapeptide tag from

the protein.

19. An antibody which recognises a hexapeptide according to any one of Claims 1 to 3.

20. An antibody according to Claim 19 which is a monoclonal antibody.

21. An antibody according to Claims 19 or 20, conjugated to an enzyme or a signal system.

22. An affinity chromatography support in which an antibody according to any one of Claims 19 to 21 is coupled to a solid support.

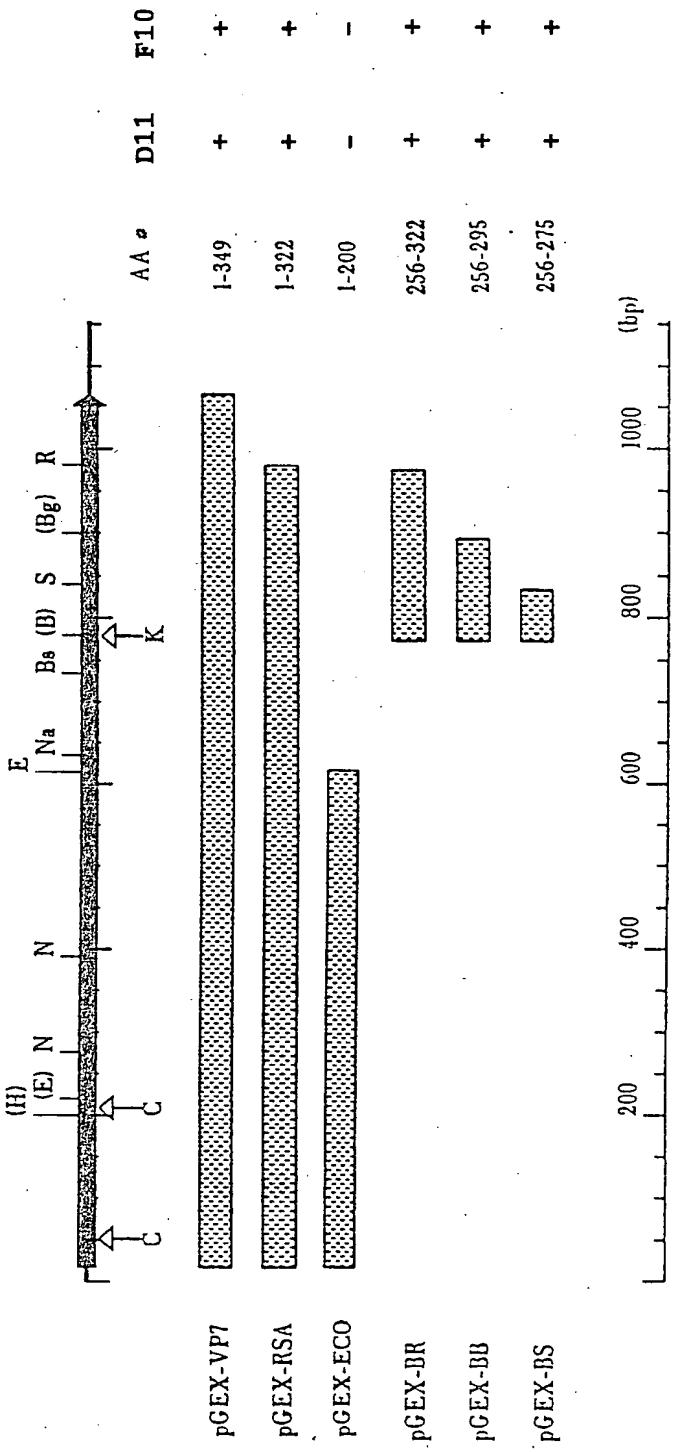
23. A kit comprising a peptide according to any one of Claims 1 to 3, and an antibody according to any one of Claims 19 to 21.

24. A kit comprising an expression vector according to Claim 8 and an antibody according to any one of Claims 19 to 21.

25. A kit according to Claims 23 or 24, wherein the antibody is present on an affinity chromatography support according to Claim 22.

26. A kit comprising a plurality of epitope-tagged molecules, wherein said tagged molecules are a series of molecular weight standards or markers.

27. A kit according to Claim 26, wherein the epitope-tag is a peptide having the sequence QYPALT.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1-322
1-200
256-322
256-295
256-275
+
+
-
+
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+
+
+
-
+
+
+
pGEX-VP7
pGEX-RSA
pGEX-ECO
pGEX-BR
pGEX-BB
pGEX-BS
(H)
(E)
N
N
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800
1000
(bp)

FIGURE 1

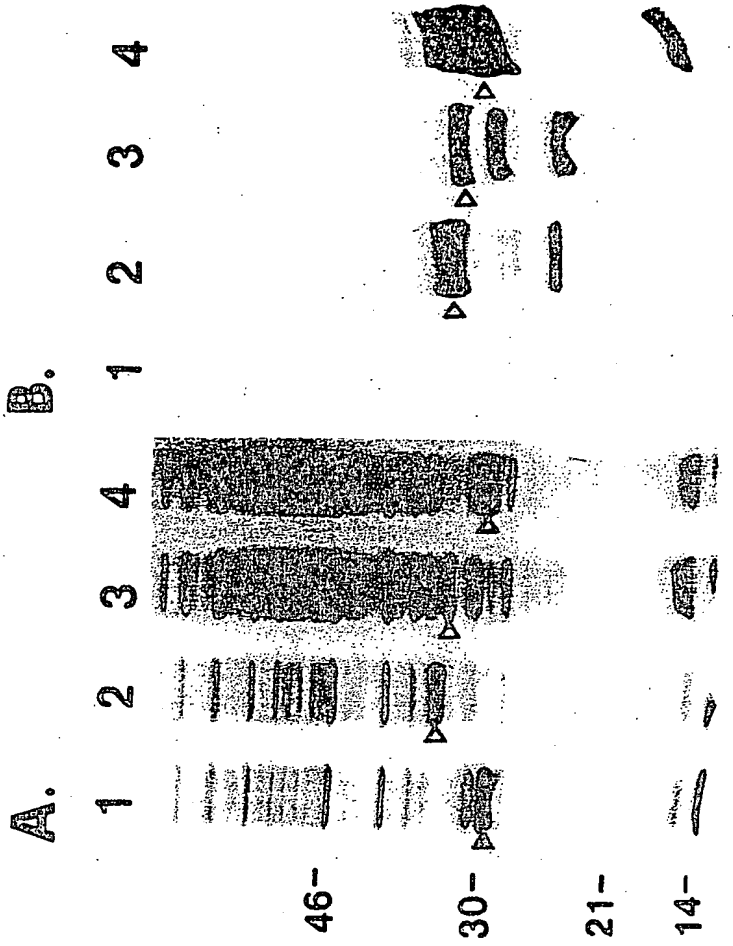

FIGURE 2

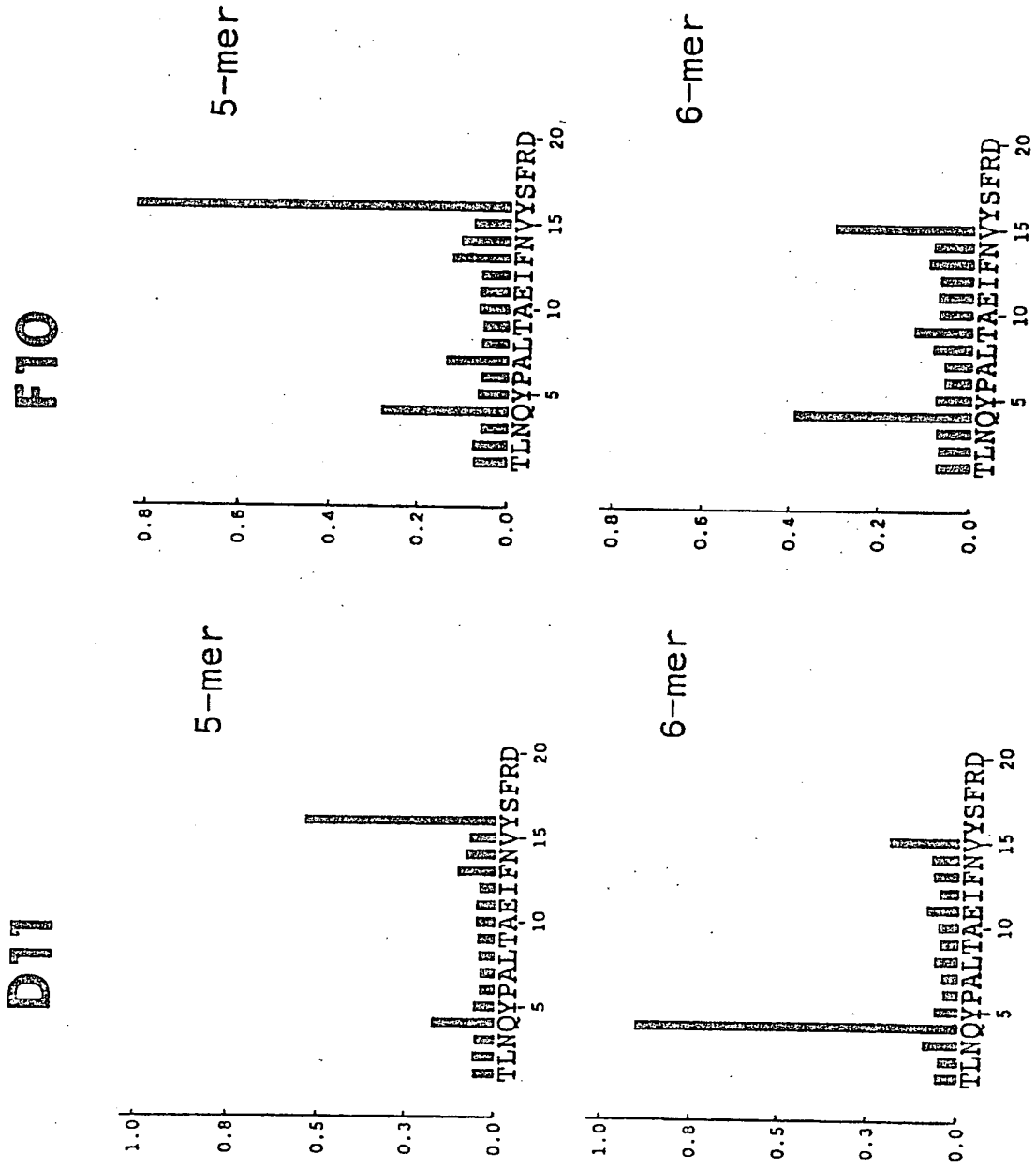

FIGURE 3
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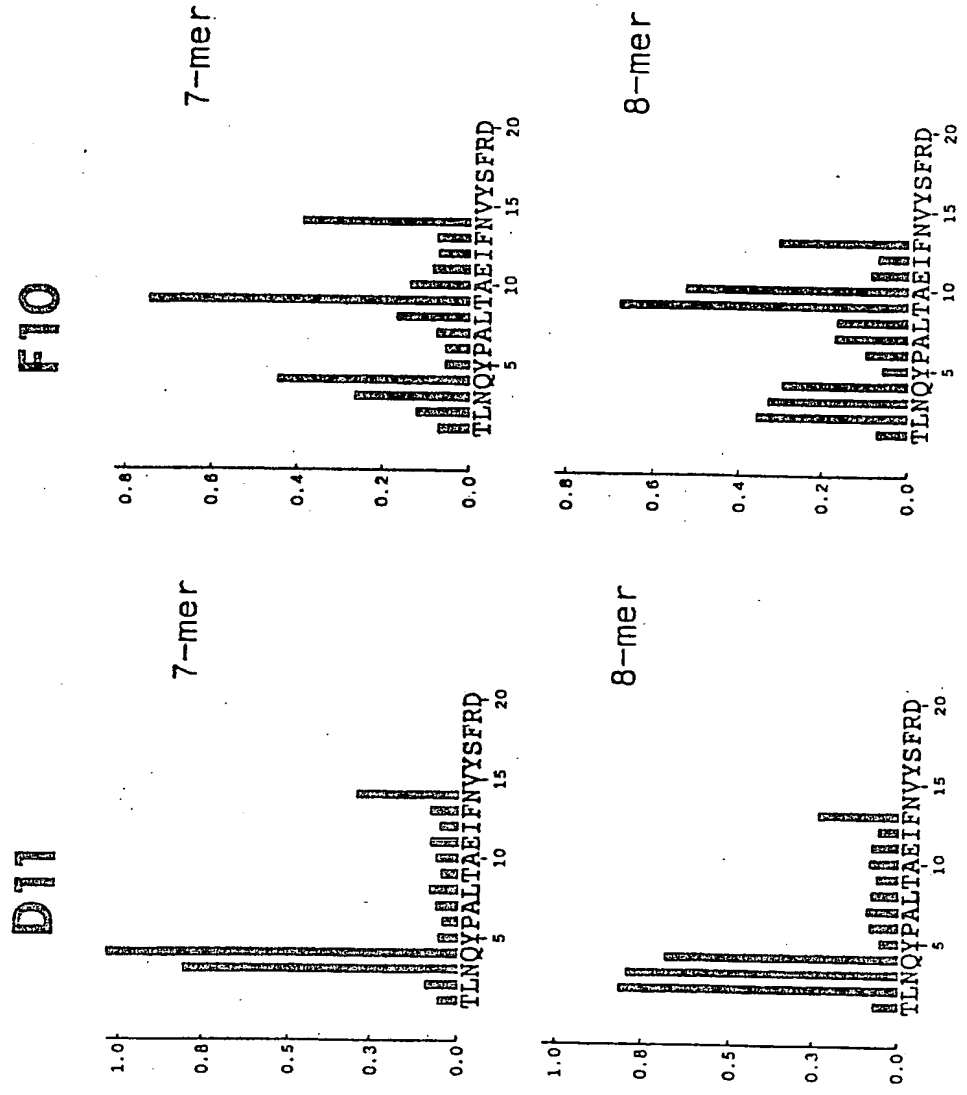

FIGURE 3 (continued)

D11/F10 Epitope MCS

```
                S  D  Q  Y  P  A  L  T  E  L  K  L  G  S  P  G  I  H
pGD1  ...TCG GAT CAA TAC CCA GCT TTG ACT GAG CTC AAG CTT GGA TCC CCG GGA ATT CAT...
                                          SacI   HindIII BamHI SmaI  EcoRI

                S  D  Q  Y  P  A  L  T  G  A  Q  A  W  I  P  G  N  S
pGD2  ...TCG GAT CAA TAC CCA GCT TTG ACT GGA GCT CAA GCT TGG ATC CCC GGG AAT TCA T...
                                          SacI   HindIII BamHI  SmaI EcoRI

                S  D  Q  Y  P  A  L  T  A  S  S  S  L  D  P  R  E  F
pGD3  ...TCG GAT CAA TAC CCA GCT TTG ACT GCG AGC TCA AGC TTG GAT CCC CGG GAA TTC AT...
                                          SacI   HindIII BamHI  SmaI  EcoRI


                R  D  Q  Y  P  A  L  T  E  L  K  L  G  S  E  F
pTD1  ...CGG GAT CAA TAC CCA GCT TTG ACT GAG CTC AAG CTT GGA TCC GAA TTC...
                                          SacI   HindIII BamHI  EcoRI

                R  D  Q  Y  P  A  L  T  G  A  Q  A  W  I  R  I
pTD2  ...CGG GAT CAA TAC CCA GCT TTG ACT GGA GCT CAA GCT TGG ATC CGA ATT C...
                                          SacI   HindIII BamHI  EcoRI

                R  D  Q  Y  P  A  L  T  A  S  S  S  L  D  P  N  S
pTD3  ...CGG GAT CAA TAC CCA GCT TTG ACT GCG AGC TCA AGC TTG GAT CCG AAT TC...
                                          SacI   HindIII BamHI  EcoRI


                L  D  Q  Y  P  A  L  T  E  L  K  L  G  S  A  A  V  D  C
pYD1  ...TTG GAT CAA TAC CCA GCT TTG ACT GAG CTC AAG CTT GGA TCC GCA GCT GTC GAC TGC AG...
                                          SacI   HindIII BamHI   PvuII  SalI  PstI

                L  D  Q  Y  P  A  L  T  G  A  Q  A  W  I  R  S  C  R  L  Q
pYD2  ...TTG GAT CAA TAC CCA GCT TTG ACT GGA GCT CAA GCT TGG ATC CGC AGC TGT CGA CTG CAG...
                                          SacI   HindIII BamHI   PvuIII SalI  PstI

                L  D  Q  Y  P  A  L  T  A  S  S  S  L  D  P  Q  L  S  T  A
pYD3  ...TTG GAT CAA TAC CCA GCT TTG ACT GCG AGC TCA AGC TTG GAT CCG CAG CTG TCG ACT GCA G...
                                          SacI   HindIII BamHI    PvuII SalI  PstI
```

FIGURE 4
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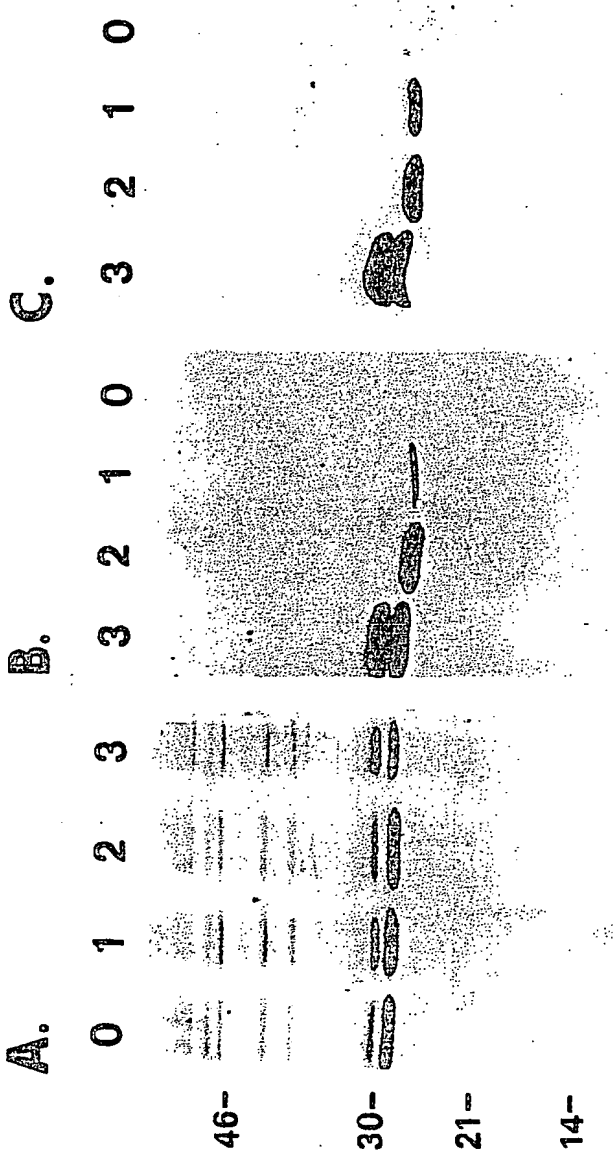

FIGURE 5

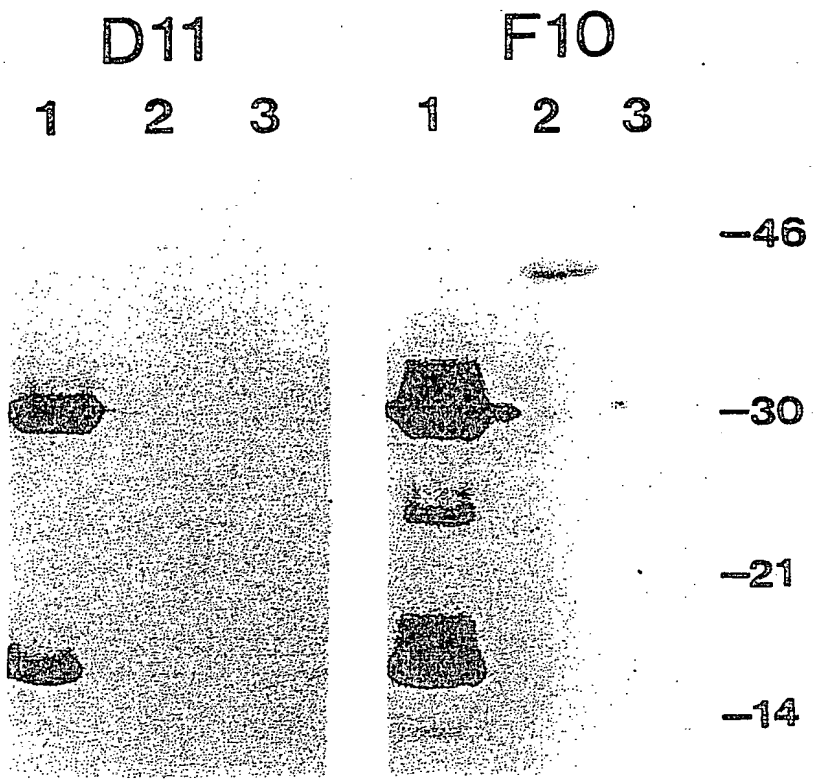

FIGURE 6

```
BAU1VP7    MDTIAARALTVMRACATLQEARIVLEANVMEILGIAINRYNGLTLRGVTM  ( 50)
BAU0VP7    ..................................................  ( 50)
BAU15VP7   ............S.V...D...............................  ( 50)

BAU1VP7    RPTSLAQRNEMFFMCLDMMLSAAGINVGPISPDYTQHMATIGVLATPEIP  (100)
BAU0VP7    ........................................T.........  (100)
BAU15VP7   ....Q..K.D.............L.......Q...G.........V.  (100)

BAU1VP7    FTTEAANEIARVTGETSTWGPARQPYGFFLETEETYQPGRWFMRAAQAVT  (150)
BAU0VP7    ..................................V...............  (150)
BAU15VP7   ....C...V...A...........A......S..VF.........P.....  (150)

BAU1VP7    AVVCGPDMIQVSLNAGARGDVQQIFQGRNDPMMIYLVWRRIENFAMAQGN  (200)
BAU0VP7    ......N..............M.......................P...  (200)
BAU15VP7   P.I.........M.....A.I.............II.....I..N....  (200)

BAU1VP7    SQQTLAGVTVSVGGVDMRAGRIIAWDGQAALQIHNPTQQNAMVQIQVVFY  (250)
BAU0VP7    ............................R.....................  (250)
BAU15VP7   .V..Q......I...NI.....T.........NV....AA...I.......  (250)

BAU1VP7    ISMDKTLNQYPALTAEIFNVYSFRDHTWHGLRTAILNRTTLPNMLPPIFP  (300)
BAU0VP7    ...........S.........................K.............  (300)
BAU15VP7   V............AS..Y.I.....P...A..M.........GV.......  (300)

BAU1VP7    PNDRDSILTLLLLSTLADVYTVLRPEFAIHGVNPMSGPLTRATARAAYV  (349)
BAU0VP7    ..................................P......I.......  (349)
BAU15VP7   .A..E.VIV.............K...I....A.IV.QI...I.......  (349)
```

FIGURE 7

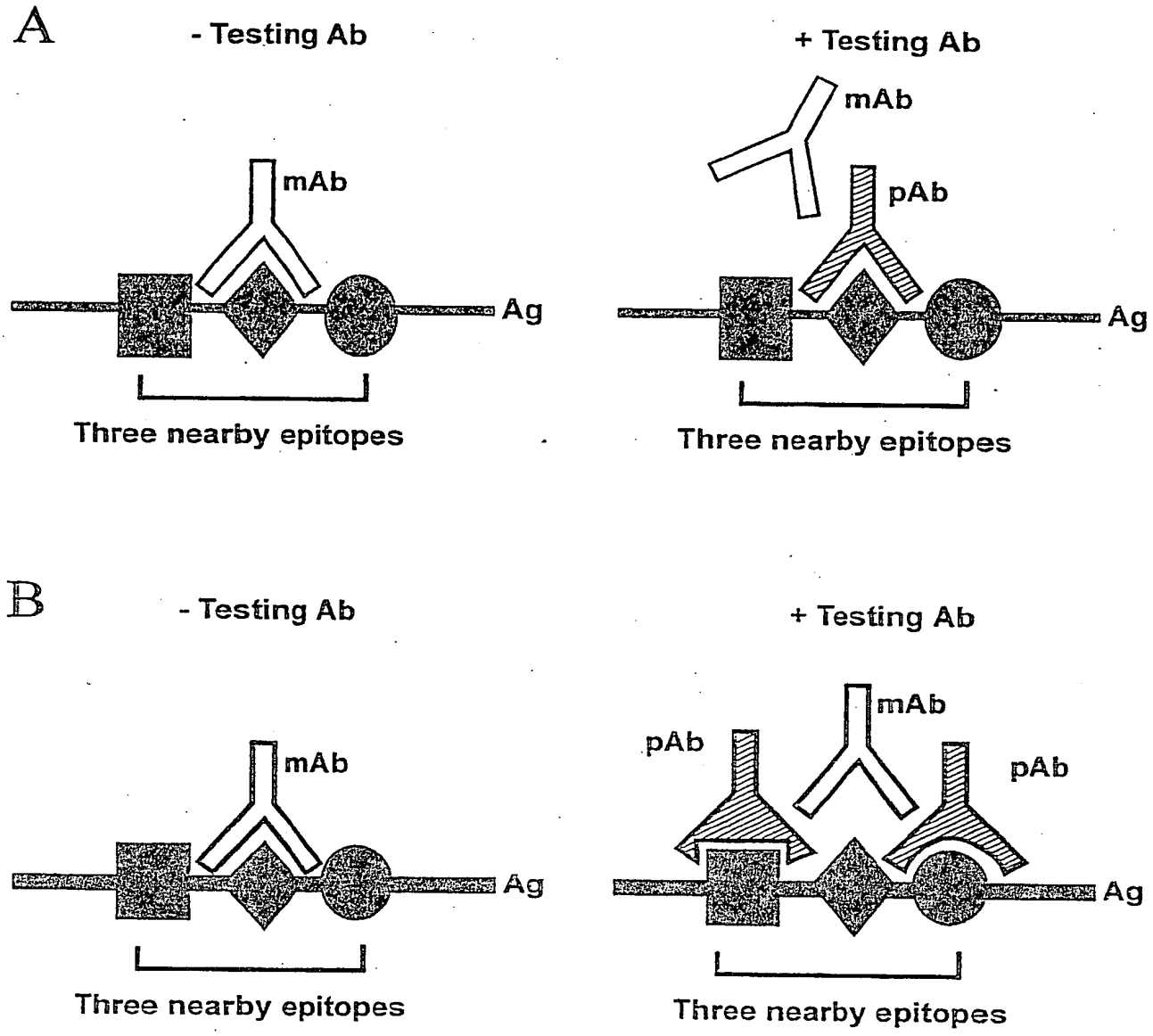
A
- Testing Ab
mAb
Ag
Three nearby epitopes
+ Testing Ab
mAb
pAb
Ag
Three nearby epitopes
B
- Testing Ab
mAb
Ag
Three nearby epitopes
+ Testing Ab
mAb
pAb
pAb
Ag
Three nearby epitopes

FIGURE 8

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 96/00516

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C07K 7/06, 1/22, 16/44; C12N 15/62; G01N 33/532

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Sequence Search : Q[YFHW] P[ASV] [LV] [TLVQ]

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Chemical Abstracts via STN : Sequence Search as above

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Gene (1996), 169 (1), "B Tag: a novel six-residue epitope tag for surveillance and purification of recombinant proteins", Wang, Lin-Fa et al, pp 53-8 Whole document	1-27
A	Virology (1994), 204(2), "Fine mapping of a surface-accessible, immunodominant site on the bluetongue virus major core protein VP7, Wang, Lin-Fa et al, pp 811-14 Whole Document	
X	Virology (1994), 198(1), "Fine mapping of a continuous epitope on VP7 of bluetongue virus using overlapping synthetic peptides and a random epitope library", Wang, Lin-Fa et al, pp346-9 Whole Document	1-27

☐ Further documents are listed in the continuation of Box C ☐ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 September 1996	9 October 1996
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (06) 285 3929	Authorized officer BARRY SPENCER Telephone No.: (06) 283 2284

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